

**FLT3 INHIBITORS IN THE TREATMENT OF FLT3/ITD AML:
OVERCOMING RESISTANCE AND DEFINING A THERAPEUTIC INDEX**

by
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Abstract

Mutations of the type III receptor tyrosine kinase (RTK) FLT3 occur in approximately 30% of acute myeloid leukemia (AML) patients and lead to constitutive activation. This has made FLT3 activating mutations an attractive drug target, as they are probable driver mutations of this disease. As more potent FLT3 inhibitors are developed, a predictable development of resistance-conferring point mutations, commonly at residue D835, has been observed. Crenolanib is a highly selective and potent FLT3 tyrosine kinase inhibitor (TKI) with activity against the internal tandem duplication (FLT3/ITD) mutants as well as against the FLT3/D835 point mutants. We tested crenolanib against a panel of D835 mutant cell lines and primary patient blasts and observed superior cytotoxic effects when compared to other available FLT3 TKIs such as quizartinib and sorafenib. Another potential advantage of crenolanib is its reduced inhibition of c-Kit compared to quizartinib. In progenitor cell assays, crenolanib was less disruptive of erythroid colony growth, which may result in relatively less myelosuppression than quizartinib. Finally, correlative data from an ongoing clinical trial demonstrate that AML patients can achieve sufficient levels of crenolanib to inhibit both FLT3/ITD and resistance-conferring FLT3/D835 mutants in vivo. Crenolanib is thus an important next-generation FLT3 TKI.

Several small molecule tyrosine kinase inhibitors (TKIs) used to treat hematologic malignancies have off target effects against c-Kit, a receptor tyrosine kinase required for normal hematopoiesis. In animal models, abnormal c-Kit signaling results in impaired erythroid and megakaryocyte production. AML patients taking FLT3 inhibitors with activity against c-Kit experience myelosuppression. To investigate the relationship

between c-Kit inhibition and myelosuppression, a panel of FLT3, c-Kit, and dual inhibitors were investigated. The activity of each drug was determined against FLT3 and c-Kit and on hematopoietic colony formation. Potent c-Kit inhibitors such as dasatinib, pazopanib, and quizartinib demonstrated the greatest disruption of erythroid colony formation. Sorafenib, which has negligible activity against c-Kit, demonstrated minimal disruption of normal colony formation. Our data highlights the importance of determining a therapeutic index between the targeted receptor and c-KIT for TKIs used to treat hematologic malignancies in order to maintain normal hematopoiesis and improve overall treatment outcomes.

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Chapter 1

Introduction

FLT3 mutated AML

Approximately one quarter of AML patients harbor an internal tandem duplication (ITD) in the receptor tyrosine kinase (RTK) FLT3 (Figure 1).¹ Point mutations of FLT3 at aspartate 835 (D835) are also observed in patients, although at a frequency of only about 7%. The FLT3/ITD mutations are known to confer a poor prognosis, while the prognostic impact of the D835 mutations is more controversial.¹⁻³ Both types of mutations lead to constitutive activation of the tyrosine kinase function, which makes FLT3 an attractive drug target to improve outcomes for AML patients with FLT3 mutations.

Clinical efficacy of tyrosine kinase inhibitors against FLT3 point mutants in AML

Over the past decade, several TKIs targeting FLT3 have been studied in the setting of clinical trials to treat AML with limited success.⁴ More recently, sorafenib and quizartinib have emerged as more potent FLT3 inhibitors and have significant clinical activity.⁵⁻⁸ Quizartinib in particular has been associated with high bone marrow response rates in relapsed and refractory FLT3/ITD AML patients, although there appeared to be a degree of myelosuppression in some patients, possibly the result of concomitant inhibition of c-KIT.^{8,9}

The BCR-ABL inhibitor imatinib has been in widespread use for CML and Ph+ ALL for a decade now. Soon after its introduction into this patient population, resistance-conferring point mutations in the ABL kinase domain emerged during therapy, leading to disease progression. This has been successfully countered with the introduction of second-generation BCR-ABL inhibitors such as nilotinib and dasatinib. In contrast, as clinical trials of FLT3 inhibitors were being conducted over the past decade, resistance-conferring point mutations in the FLT3 coding sequence were not routinely observed, except sporadically.¹⁰ A reasonable explanation for this is that high-level FLT3 inhibition in vivo was not generally achieved by the first generation FLT3 inhibitors. However, as the more potent inhibitors sorafenib and quizartinib have been more

widely used to treat FLT3/ITD AML, both in clinical trials and in off-label use, point mutations have finally emerged during disease progression.¹¹ These mutations are predominantly found at aspartate 835 (D835) in the activation loop, although mutations at phenylalanine 691 (F691) were also reported. Both mutations render the receptor resistant to the FLT3 inhibitor to a variable degree and are clearly associated with disease progression. Resistance-conferring FLT3 D835 mutations have also been reported to arise during sorafenib and sunitinib therapy.^{12,13} As the clinical development of FLT3 inhibitors proceeds into advanced phase trials, it has become obvious that these mutations will represent a new obstacle in the care of FLT3/ITD AML patients, at least when quizartinib or sorafenib are used as the initial TKI. Furthermore, FLT3/D835 mutations present at diagnosis in 7% of AML patients and in some cases may represent a driver of the disease.¹⁴⁻¹⁶ While first generation FLT3 inhibitors such as midostaurin have activity against FLT3 tyrosine kinase domain mutations,^{17,18} their relative lack of potency has limited their utility as single agents.

Crenolanib is a benzimidazole quinolone derivative originally developed as an inhibitor of platelet-derived growth factor receptor (PDGFR).¹⁹ In a phase 1 trial of crenolanib enrolling solid tumor patients, eight patients achieved stable disease but no objective responses were reported, despite micromolar plasma concentrations being achieved in tolerable fashion.¹⁹ Subsequent analysis using an in vitro kinase assay indicated that the compound had activity against FLT3. We report here the characterization of crenolanib as a novel TKI that has potent activity against both the FLT3/ITD-mutated receptor as well as to FLT3/D835-mutated receptors, including those arising in the context of FLT3/ITD mutations following treatment with sorafenib and quizartinib.

Mechanisms of resistance to tyrosine kinase inhibitors in AML

There are many additional mechanisms of resistance to TKIs in AML beyond acquired mutations in the FLT3 receptor itself. First, this constantly growing collection of FLT3 TKIs includes a wide variety of drugs with highly variable pharmacokinetic properties. All are

metabolized by CYP3A4 enzymes and therefore their pharmacokinetics are altered by concomitant administration of other CYP3A4-metabolized medications. Variable degrees of plasma protein binding can greatly affect the efficacy of these drugs.²⁰ Patients, especially those receiving chemotherapy, can have widely different concentrations of plasma FL which has also been demonstrated to decrease efficacy of FLT3 TKIs.²¹ It has also been shown that these blasts will overcome their addiction to FLT3 signaling by activating parallel pathways including upregulation of various survival, proliferation, and anti-apoptotic molecules.²² AML is also a clonal disease, with various subpopulations constantly evolving in response to treatment. The complexity of these issues has made it difficult to combat this issue of TKI resistance in AML.

A particularly interesting and complex form of resistance is the contribution of the tumor microenvironment. The bone marrow niche is well documented as a protective environment for both normal hematopoietic cells and malignant cells. AML therapy is complicated by this stromal protection as it only takes only a small amount of residual leukemia stem cells surviving after the treatment to result in disease relapse. We have demonstrated with a primary human bone marrow and primary human leukemia blast co-culture system that bone marrow stroma confers on leukemia cells a protective effect against FLT3 TKIs. The exact mechanism of this phenomenon is not yet clear but there are a few important details recently discovered. TKIs are capable of inducing apoptosis in peripheral blasts but induce a pathway of differentiation rather than death in blasts on stroma.²³ It has also been shown that stimulation of the MAPK pathway contributes to enhanced viability of blasts on stroma.²⁴ Finally, the addition of FL is known to reduce the efficacy of TKIs in cell suspension.²¹ We hypothesized that stromal surface FL is playing a role in resistance and anti-FL mAbs may alleviate this stromal cell-mediated resistance to TKIs.

c-Kit and myelosuppression

Myelosuppression is a common adverse event noted during the development of new agents for patients with leukemia. Distinguishing between disease-related effects and the effects of the therapy can be a challenge. Tyrosine kinase inhibitors represent a rapidly expanding new class of anti-cancer therapy. Many TKIs have activity against c-KIT, a receptor tyrosine kinase which is essential for normal erythropoiesis and megakaryocyte function.²⁵ Patients treated with TKIs that inhibit c-KIT, therefore, would be predicted to experience myelosuppression.²⁶ In vivo c-KIT inhibition is also associated with hair depigmentation, which can be a useful biomarker for target inhibition (Figure 2).²⁷ However, drugs such as pazopanib and sunitinib, which have in vitro and in vivo activity against c-KIT, are not reported as inducing myelosuppression in solid tumor patients. In contrast, dasatinib and imatinib, which are reported to have in vitro activity against c-KIT, can readily induce myelosuppression in patients with Ph+ acute lymphocytic leukemia (Ph+ ALL) or chronic myeloid leukemia (CML).^{28,29} In patients with relapsed/refractory FLT3/ITD AML, treatment with the novel FLT3 inhibitor quizartinib was associated with myelosuppression, whereas in a similar patient population, a different FLT3 inhibitor, sorafenib, induced no myelosuppression.^{6,30} To better understand the relationship between inhibition of c-KIT, FLT3, and marrow suppression, we studied a series of different TKIs using normal bone marrow progenitor assays and dose-response experiments with activated, phosphorylated c-kit. Our findings highlight the importance of determining a therapeutic index between the targeted receptor and c-KIT. This is an important consideration in the development of TKIs for patients with primary marrow disorders such as leukemia.

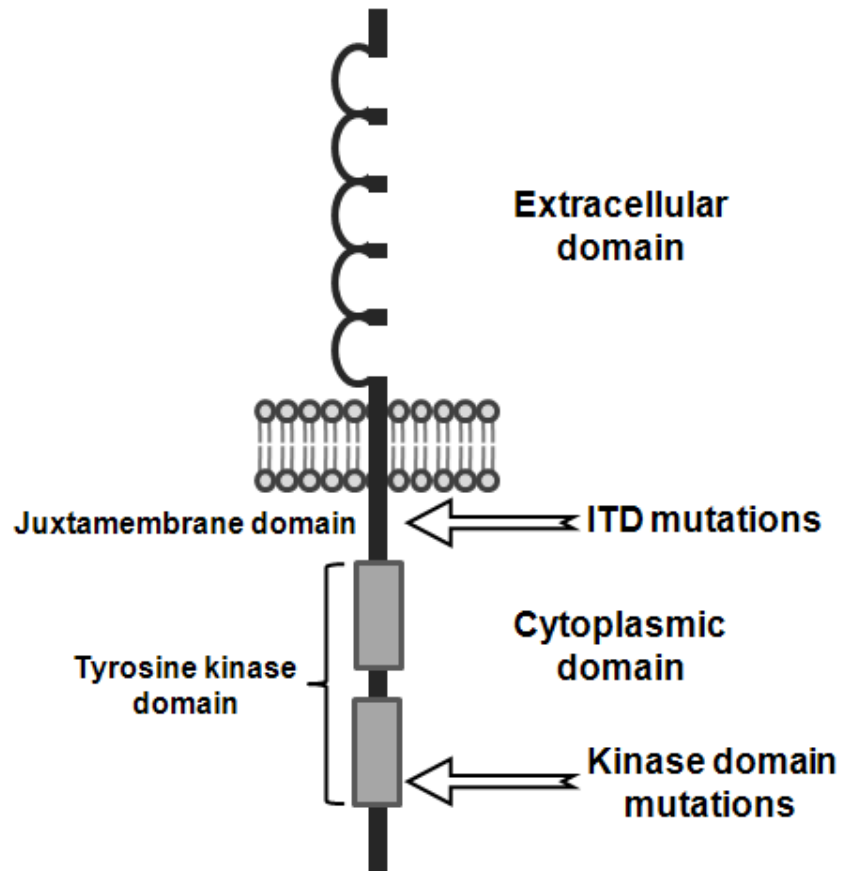


Figure 1. Diagram of the FLT3 receptor. The FLT3 receptor has five extracellular immunoglobulin domains followed by a transmembrane domain. Its cytoplasmic domain includes a juxtamembrane domain (the location of the internal tandem duplication mutation) followed by a split kinase domain (the location of the D835 residue where kinase domain mutations commonly occur).



Figure 2. TKI induced hair depigmentation. An AML patient before treatment (left) and 54 days after treatment (right) with the c-Kit/FLT3 inhibitor, PLX3397. (This compound is currently being studied in a phase 1 trial- see NCT01349049; pharmacokinetic data are not yet available for PLX3397, and so it was not included in this study).

Chapter 2

Crenolanib is a potent inhibitor of FLT3/ITD and FLT3/D835
AML

Crenolanib has activity against FLT3/ITD AML cells

Crenolanib (Figure 3A) was originally developed as an inhibitor of PDGFRB. In a commercially-available assay of kinase selectivity (KinomeScan, DiscoverX, San Diego, CA), crenolanib was demonstrated to have a high degree of selectivity for FLT3 relative to other kinases (Figure 3B). We compared the FLT3 inhibitory activity of crenolanib with sorafenib and quizartinib using cell lines expressing mutant and wild type FLT3 (Figure 4). In immunoblot assays examining FLT3 autophosphorylation and phosphorylation of downstream signaling proteins (Figure 4A-B), crenolanib inhibited autophosphorylation of both wild type and ITD-mutated FLT3 to a similar degree, with an IC₅₀ of approximately 2 nM. In MTT assays, crenolanib exhibited a cytotoxic effect against Molm14 and MV4-11 cells in culture medium to a similar degree as sorafenib and somewhat less so compared with quizartinib (Figure 4C). This effect was also observed in assays of apoptosis using Annexin V (Figure 4D). Likewise, crenolanib inhibited FLT3 autophosphorylation in primary FLT3/ITD AML blasts (from a patient with a FLT3/ITD mutation at an allelic ratio of 0.95) in culture with an IC₅₀ of 2.4 nM (Figure 4E).

Crenolanib has activity against FLT3/D835 point mutants

Point mutations at residue D835 can arise in the wild type FLT3 receptor or within the context of an existing ITD mutation (i.e., within the same allele). In order to test the inhibitory activity of crenolanib against both categories of D835 mutations, we generated a series of transfectants of the murine lymphoid cell line Ba/F3. As shown in Table 1, crenolanib and quizartinib have similar potency against a FLT3/ITD receptor

lacking any kinase domain mutation. However, crenolanib was significantly more potent than quizartinib against the D835Y, D835F, and D835V mutant receptors. Interestingly, crenolanib and quizartinib were of equal potency against the D835H and D835N variants. Crenolanib has minimal activity against the F691L variant.

We next tested crenolanib against a series of primary AML samples with D835 mutations arising both spontaneously and in the context of FLT3 inhibitor therapy. Figure 5A shows a dose response experiment using 3 primary AML samples in vitro, each of which harbored a D835 mutation (in a wild type FLT3 background, no ITD mutation). Patients 2 and 3 harbored D835Y mutations and were newly-diagnosed, while patient 4, harboring a D835V mutation, was collected at relapse. Crenolanib inhibited FLT3 autophosphorylation in all three samples, with IC_{50} 's of 1.2, 8.1, and 2.0 nM, respectively. No significant cytotoxicity was induced by the drug in the two diagnostic specimens, while the relapsed specimen did appear to respond (Figure 5B). This is similar to what has been observed with FLT3/ITD-mutated samples, in that newly-diagnosed cases are not generally responsive to highly selective FLT3 inhibition, while in the relapsed setting the response is more predictable.³¹ Examining this relapsed patient sample further, Figure 5C shows that crenolanib is more effective than either sorafenib or quizartinib at inhibiting FLT3 autophosphorylation and at inducing cytotoxicity. Finally, the utility of crenolanib against a tyrosine kinase domain mutation in the background of a FLT3/ITD mutation is demonstrated in Figure 5D. A FLT3/ITD AML patient (with an allelic ratio of 2.65) who was being maintained on sorafenib developed clinical progression, and was found to harbor a D835F mutation which was not present at the start of sorafenib therapy. Again, of the three inhibitors, only crenolanib induced a significant

effect against these blasts in vitro (there were insufficient blasts from this sample to perform immunoblotting).

c-Kit inhibition and effects on hematopoietic colony formation

FLT3 inhibitors often have inhibitory activity against the closely-related RTK, c-KIT. This may account to some degree for the myelosuppression, particularly the anemia and thrombocytopenia that is often observed in patients receiving FLT3 inhibitors in clinical trials. An extensive literature supports the notion that suppression of c-KIT activity would be expected to result in impaired red cell and platelet production.²⁵ A relative therapeutic index, therefore, would be expected to exist for inhibitors of class III RTKs such as crenolanib and quizartinib. We compared the activity of both drugs against c-KIT using immunoblot assays with the erythroleukemia cell line, TF-1, which expresses wild type c-KIT (Figure 6A). Quizartinib was the more potent inhibitor of c-KIT in this assay. In hematopoietic progenitor cell assays of normal donor marrow, quizartinib, but not crenolanib, was generally more suppressive of erythroid activity at concentrations consistent with those associated with c-KIT inhibition (Figure 6B). Crenolanib, therefore, has an advantage over quizartinib in this respect, in that the relative degree of myelosuppression it induces in patients may be less.

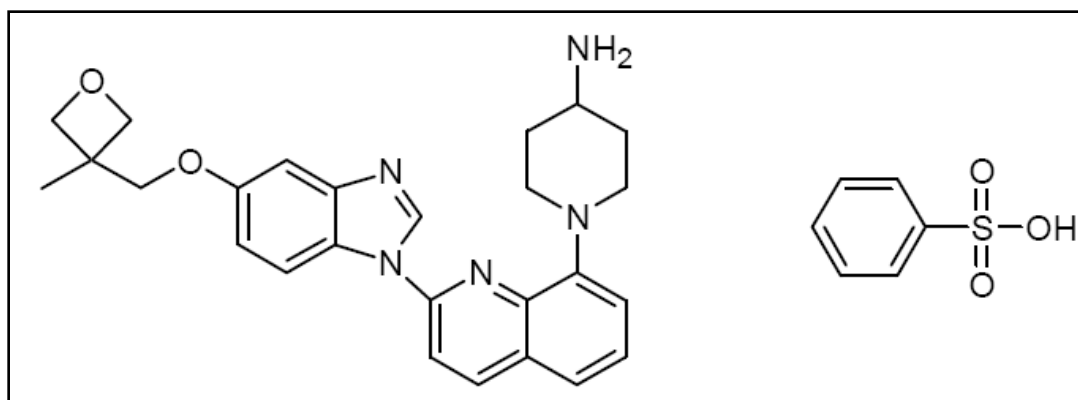


Figure 3A. Crenolanib. The structure of crenolanib.

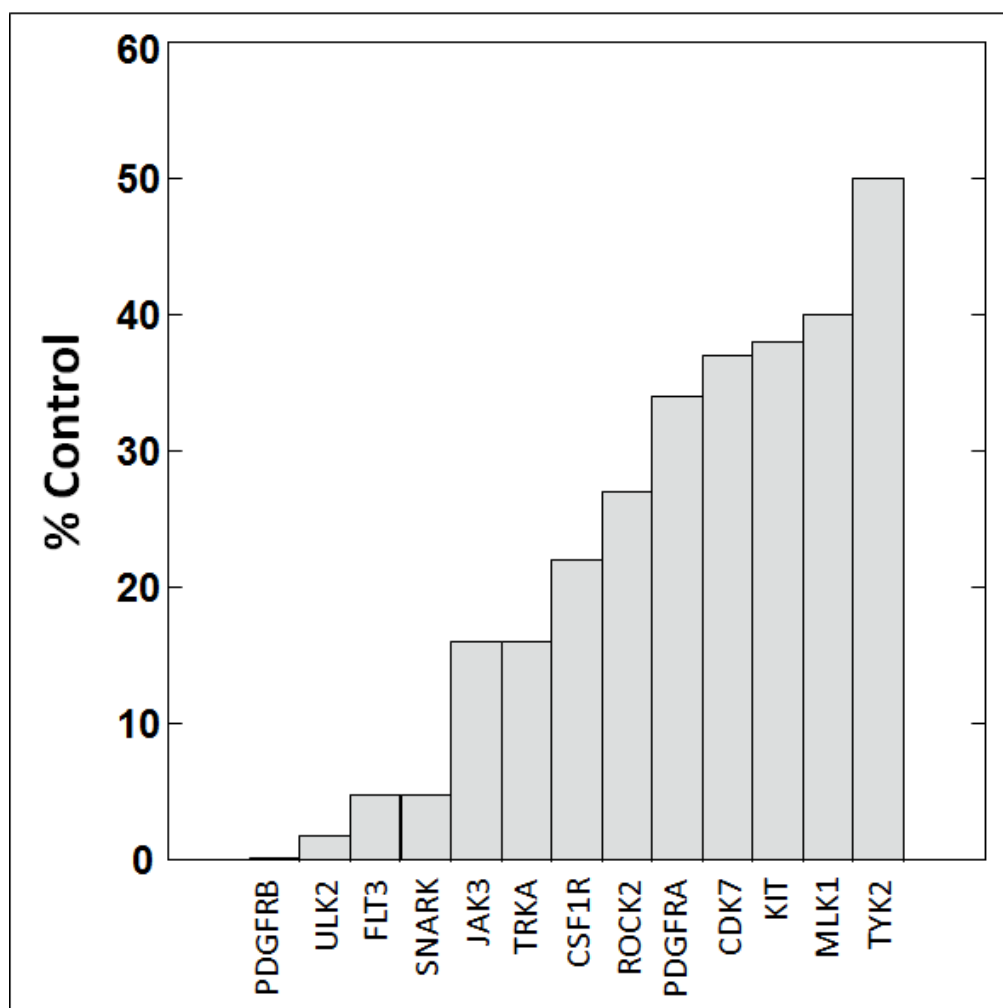


Figure 3B. KinomeScan results for crenolanib. Shown are the results of all kinases in the panel inhibited 50% or more (compared with baseline activity) by 100 nM crenolanib.

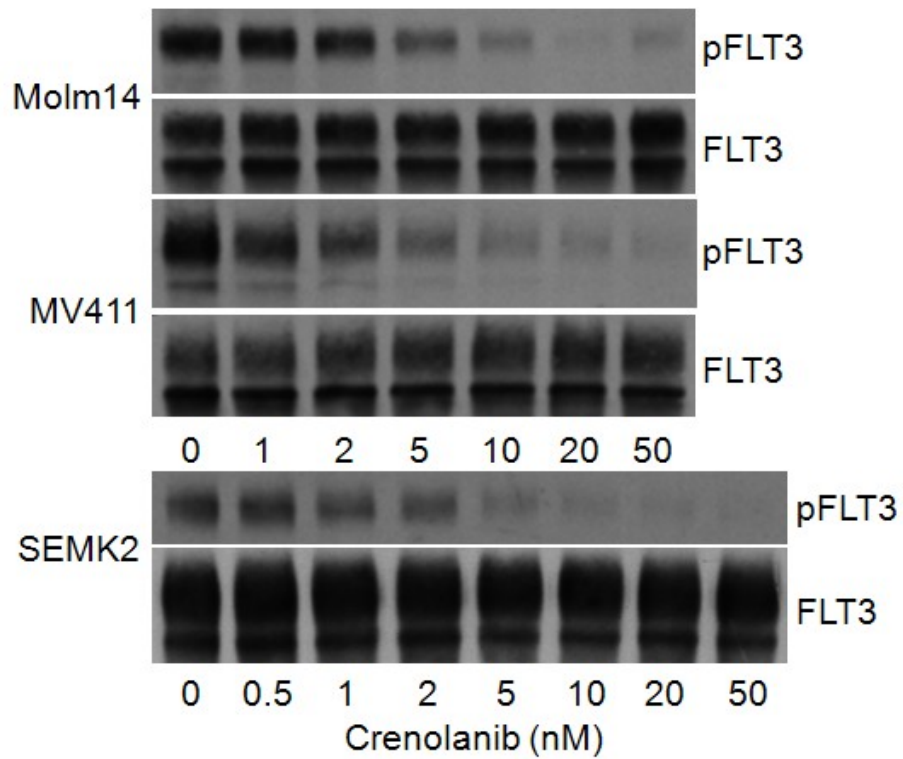


Figure 4A. Activity of crenolanib against wild type and ITD-mutated FLT3 in vitro.

FLT3/ITD cell lines (Molm14 and MV411) and FLT3 WT cell lines (SEMK2) were treated with crenolanib for one hour, then cells were lysed, immunoprecipitated for FLT3 and analyzed by immunoblotting for phospho- and total FLT3.

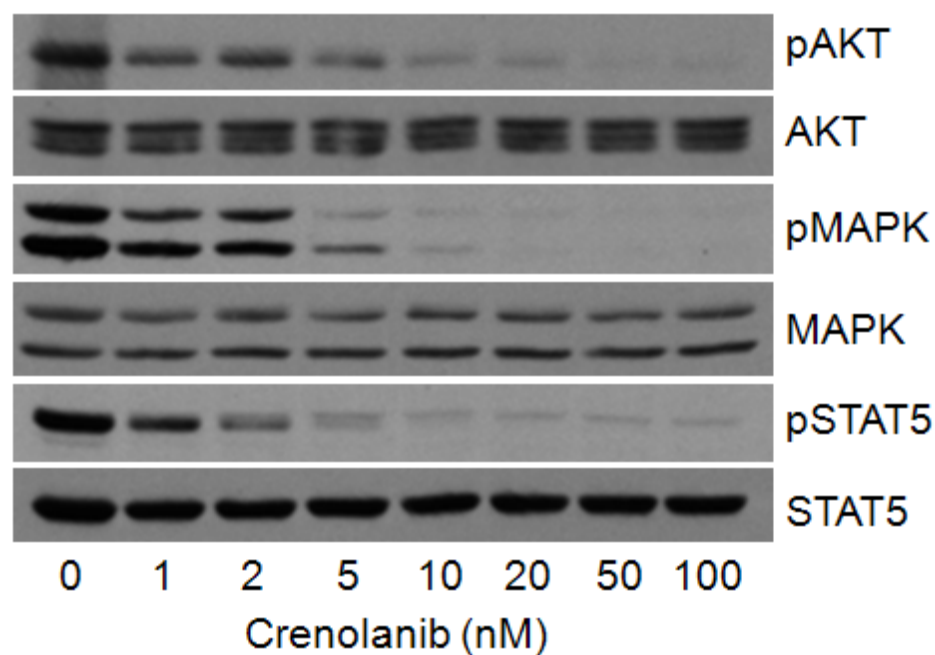


Figure 4B. Activity of crenolanib against wild type and ITD-mutated FLT3 in vitro.

An aliquot of the lysate of Molm14s treated with crenolanib from panel A was reserved for analysis of downstream signaling molecules including pAKT, pMAPK, pSTAT5, as well as the corresponding total protein content.

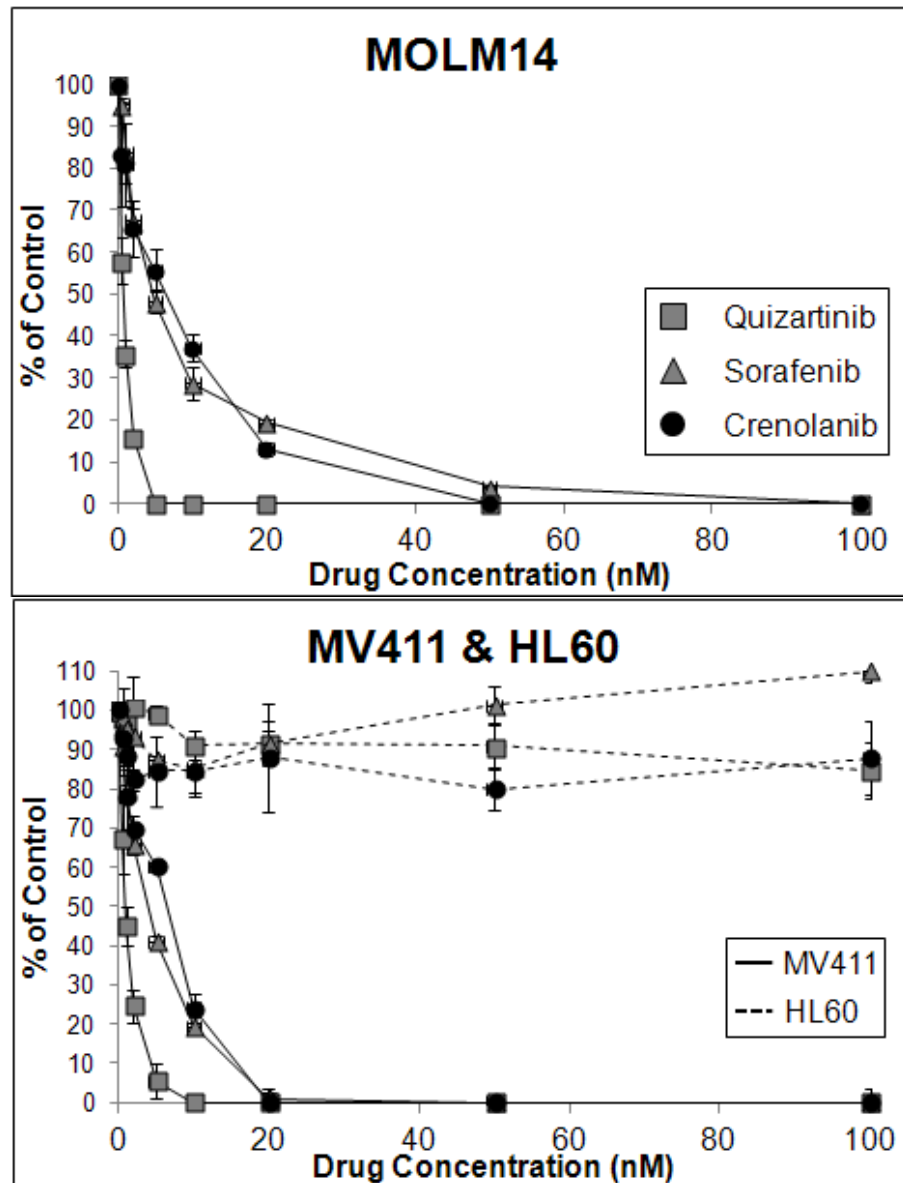


Figure 4C. Activity of crenolanib against wild type and ITD-mutated FLT3 in vitro.

Cytotoxicity of these doses of crenolanib in FLT3/ITD cell lines was analyzed by MTT assay. HL-60 cells which express extremely limited WT FLT3 were used as a control.

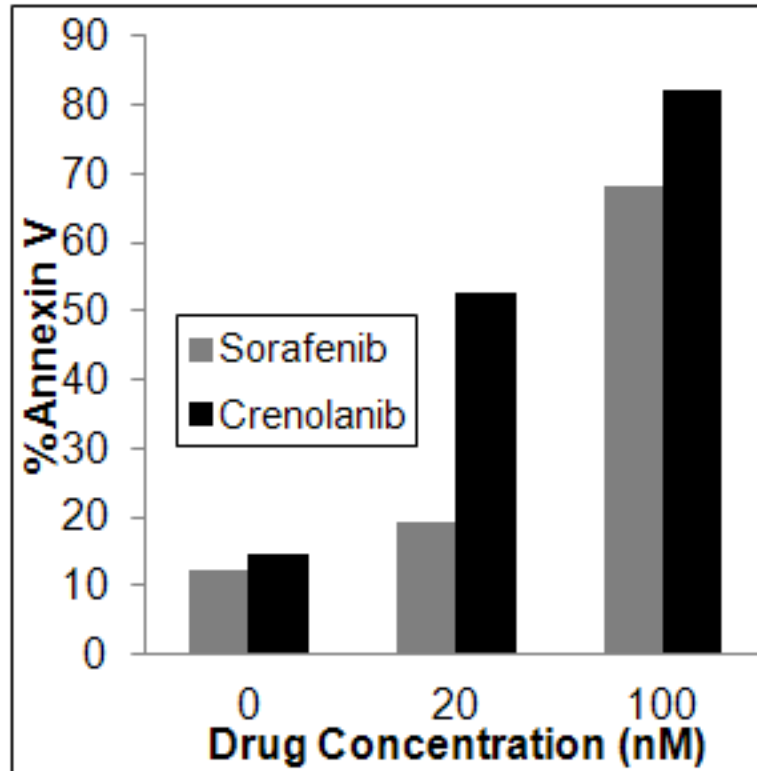


Figure 4D. Activity of crenolanib against wild type and ITD-mutated FLT3 in vitro.

Apoptosis induced by crenolanib and sorafenib 48 hrs after treatment of Molm14 cells with drug was analyzed by Annexin V flow cytometry.

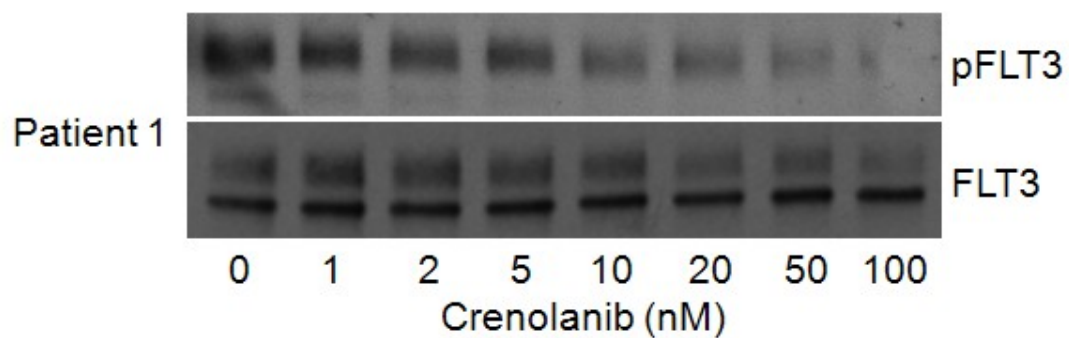


Figure 4E. Activity of crenolanib against wild type and ITD-mutated FLT3 in vitro.

Patient blasts containing a FLT3/ITD mutation were treated with crenolanib, lysed, and immunoblotted for phospho- and total FLT3.

Cell Line	<u>Crenolanib</u> IC₅₀ pFLT3	<u>Quizartinib</u> IC₅₀ pFLT3	Fold difference
Ba/F3 ITD	1.3 nM	1.7 nM	1.2
Ba/F3 ITD/D835Y	8.7 nM	93.1 nM	10.7
Ba/F3 WT D835Y	6.9 nM	33.7 nM	4.9
Ba/F3 WT D835F	6.5 nM	72.7 nM	11.2
Ba/F3 WT D835H	19.8 nM	20.0 nM	-
Ba/F3 WT D835N	4.3 nM	2.3 nM	0.5
Ba/F3 WT D835V	2.3 nM	63.7 nM	27.4
Ba/F3 ITD/F691L	67.8 nM	36.4 nM	0.5

Table 1. Inhibitory activity of crenolanib against different FLT3 TKD mutations.

Ba/F3 cell lines were treated with quizartinib or crenolanib for one hour, then cells were lysed, immunoprecipitated for FLT3 and analyzed by immunoblotting for phospho- and total FLT3. Densitometry analysis was performed using Quantity One software (version 4.5.0). IC₅₀s were calculated by regression analysis after linear conversion (Calculusyn software).

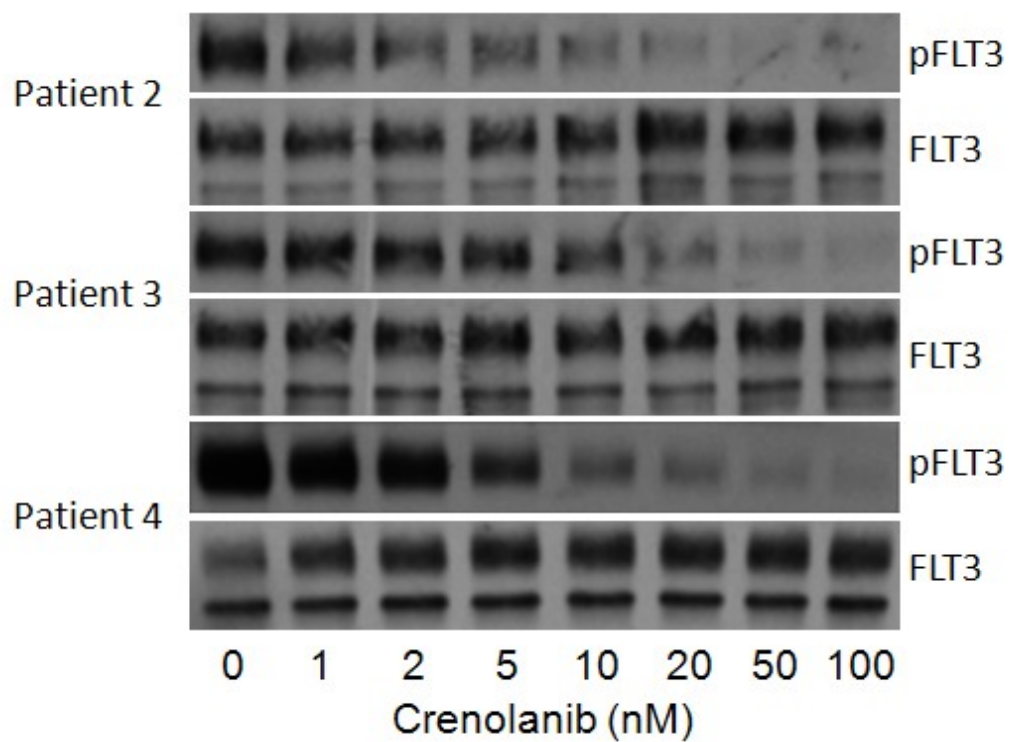


Figure 5A. Inhibitory activity of crenolanib against FLT3 TKD mutations in primary AML samples. Blasts from patient 2 (de novo D835Y), patient 3 (de novo D835Y), and patient 4 (relapsed D835V) were incubated for one hour with crenolanib, lysed, immunoprecipitated for FLT3, and analyzed for phospho- and total FLT3 by immunoblotting.

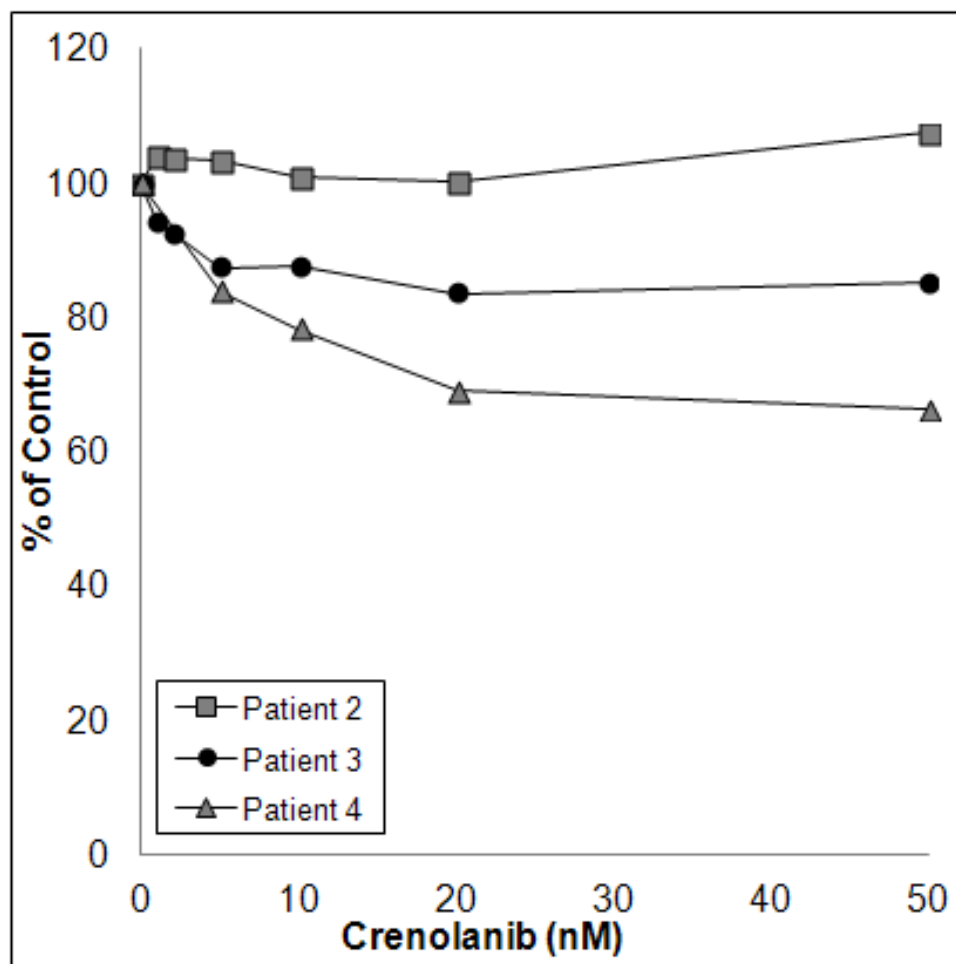


Figure 5B. Inhibitory activity of crenolanib against FLT3 TKD mutations in primary AML samples. Blasts from the same patients in Figure 5A were treated with crenolanib in 96 well plates for 72 hours and cytotoxicity was analyzed by MTT.

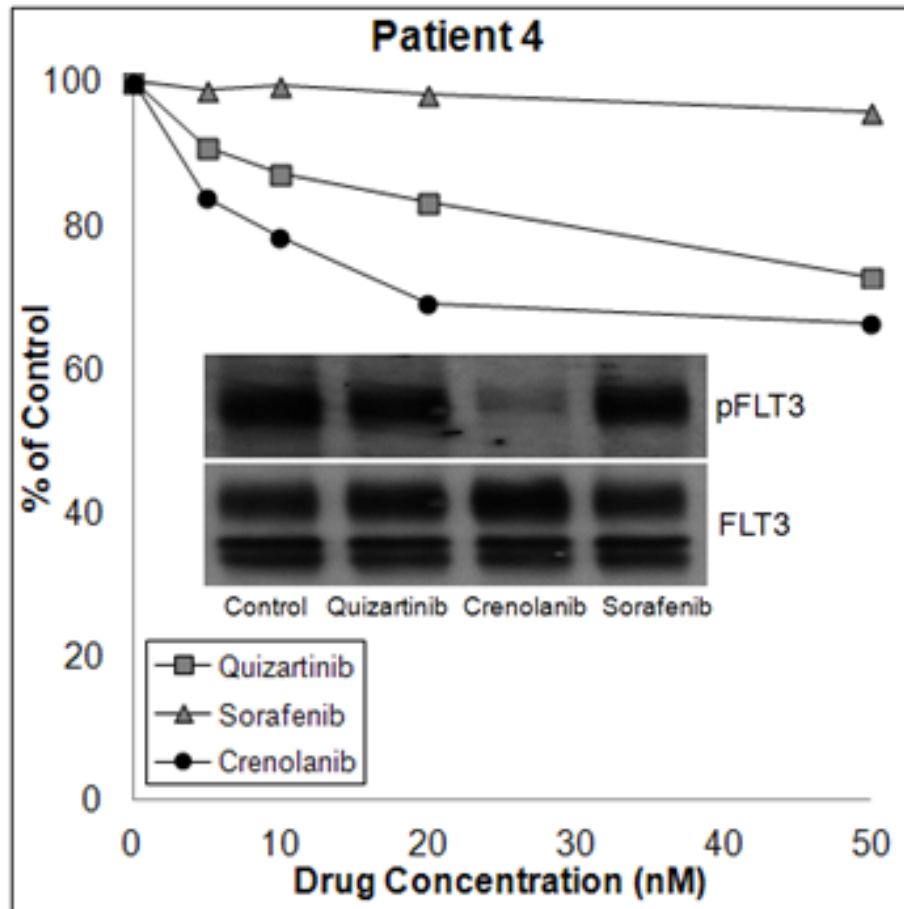


Figure 5C. Inhibitory activity of crenolanib against FLT3 TKD mutations in primary AML samples. Blasts from patient 4 were also treated with quizartinib and sorafenib in this analysis. In a separate immunoblot experiment (inset), blasts from patient 4 were treated with 20 nM of quizartinib, crenolanib, and sorafenib for one hour, lysed, immunoprecipitated for FLT3, and analyzed by immunoblot for phospho- and total FLT3.

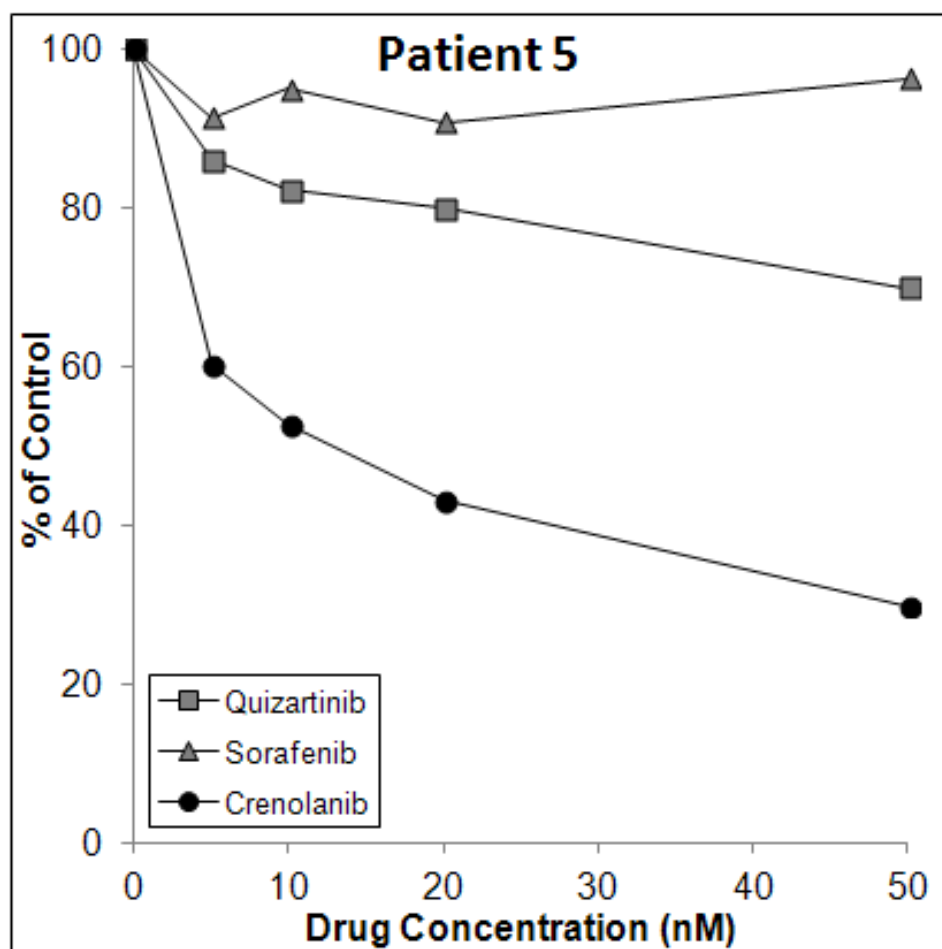


Figure 5D. Inhibitory activity of crenolanib against FLT3 TKD mutations in primary AML samples. Patient 5 had a FLT3/ITD mutation and was treated with sorafenib, responded, and then relapsed. Blasts collected after relapse harbored a D835F mutation, along with the original FLT3/ITD mutation. These blasts were treated with crenolanib, quizartinib, or sorafenib in 96 well plates for 72 hours and cytotoxicity was analyzed by MTT.

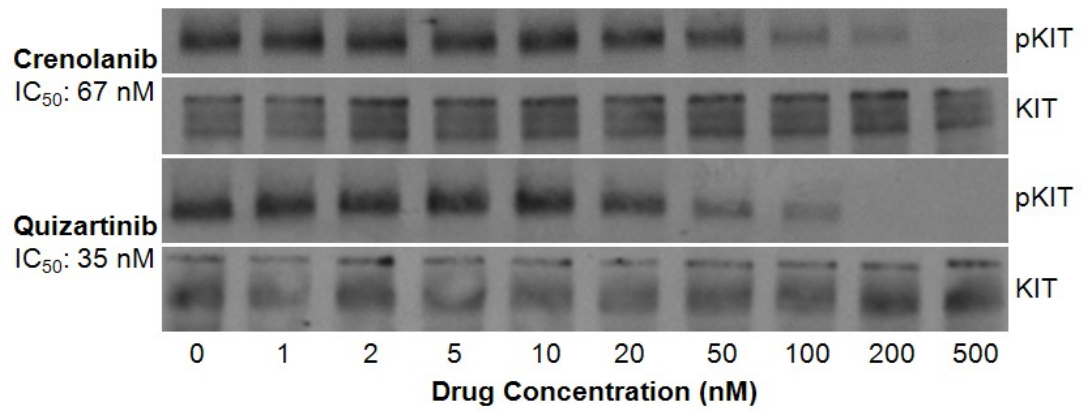


Figure 6A. Inhibition of c-KIT and erythropoiesis. TF-1 cells, which express WT c-Kit, were treated with crenolanib or quizartinib for one hour. In the last five minutes of drug treatment, 20 ng of SCF was added. The cells were lysed, immunoprecipitated for c-Kit and analyzed by immunoblot for phospho- and total c-Kit.

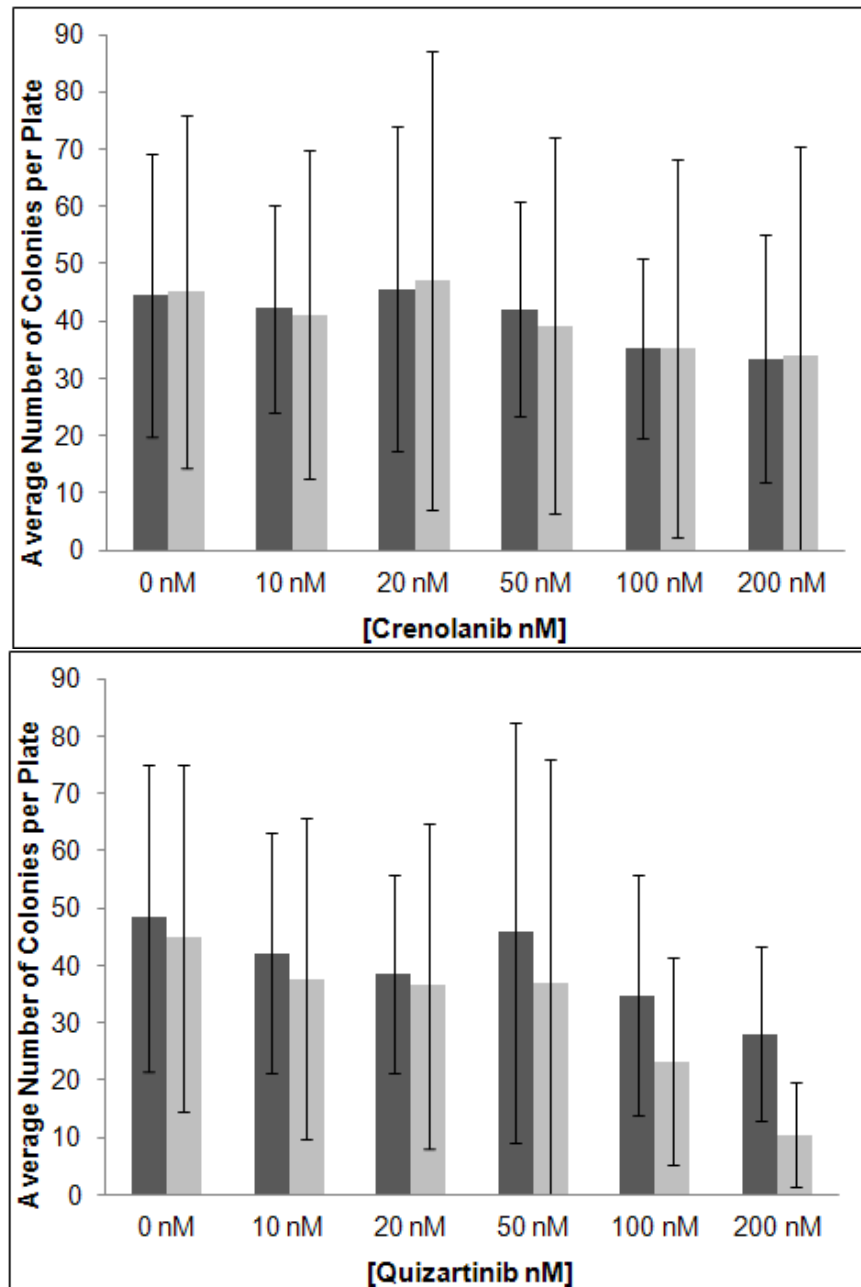


Figure 6B. Inhibition of c-KIT and erythropoiesis. Normal human donor bone marrow (n=3) was collected and diluted to a concentration of 100,000 cells per mL in MethoCult. Various concentrations of crenolanib or quizartinib were added and cells were plated in quadruplicate in 35 mm dishes. Each dish was view under a light microscope and total numbers of CFU-GM and BFU-E colonies were recorded.

Chapter 3

Clinical correlative analysis of phase II trials of crenolanib

Crenolanib achieves inhibitory activity against FLT3/ITD and FLT3/D835 AML in vivo

Crenolanib is currently being studied in a phase II clinical trial for patients with relapsed/refractory AML with FLT3 activating mutations (NCT01657682). Patients are treated with 100 mg crenolanib orally every eight hours. However, on study day 1, patients were given a single dose of drug so that single-dose pK studies could be conducted. We used plasma samples collected from patients on day 1 of treatment with crenolanib for analysis of in vivo FLT3 inhibition using the PIA assay, and pK analysis was carried out using serum from these time points to determine serum drug concentrations at the corresponding PIA time points (Figures 7A-B). No significant difference was seen between experiments performed in plasma compared to serum which showed IC₅₀s against phospho-FLT3 of 48 nM and 40 nM, respectively. Comparing the PIA results with the pK results, we observed that FLT3 inhibition was readily achieved throughout the dosing period. A trough serum concentration of 100-200 nM appears to be required to maintain inhibition of FLT3 autophosphorylation to 10-15% of baseline. This PIA assay was performed using Molm14 cells, which harbor a FLT3/ITD mutation. To provide evidence for the in vivo activity of crenolanib against kinase domain mutations, we took blasts from patient 4 (described above), which contained a D835V mutation, and used them in a PIA assay of trough plasma samples from several different trial patients. As shown in Figure 7C, steady state (Day 15) levels of crenolanib were quite sufficient to profoundly inhibit this mutant FLT3 receptor. These clinical correlative data indicate that AML patients treated with 100 mg crenolanib every eight hours are able to achieve sufficient concentrations of the drugs to effectively inhibit the

target, whether it is a FLT3/ITD mutation or a FLT3/D835 mutation. Data regarding the safety, tolerability, and efficacy of crenolanib in these patients is not yet available, as accrual is ongoing.

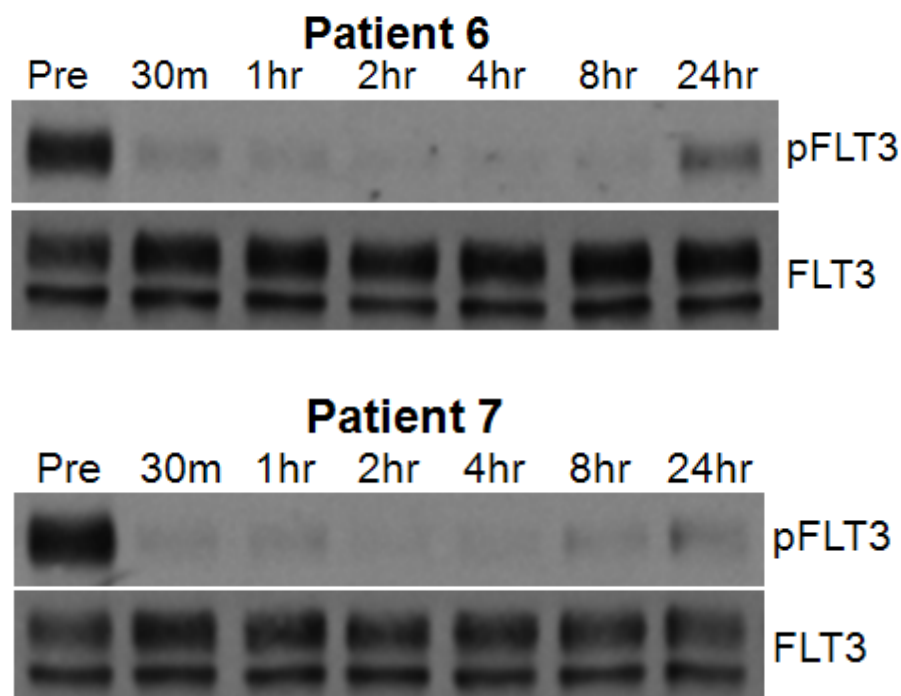


Figure 7A. Crenolanib inhibits FLT3 in vivo. Plasma samples from patient 6 and patient 7 were collected as part of the phase II clinical trial of crenolanib. Patients received a single dose of drug on Day 1, and samples were collected for PIA and pK analysis. Molm14 cells were incubated with each plasma sample for one hour, lysed, immunoprecipitated for FLT3, and analyzed by immunoblot for phospho and total FLT3.

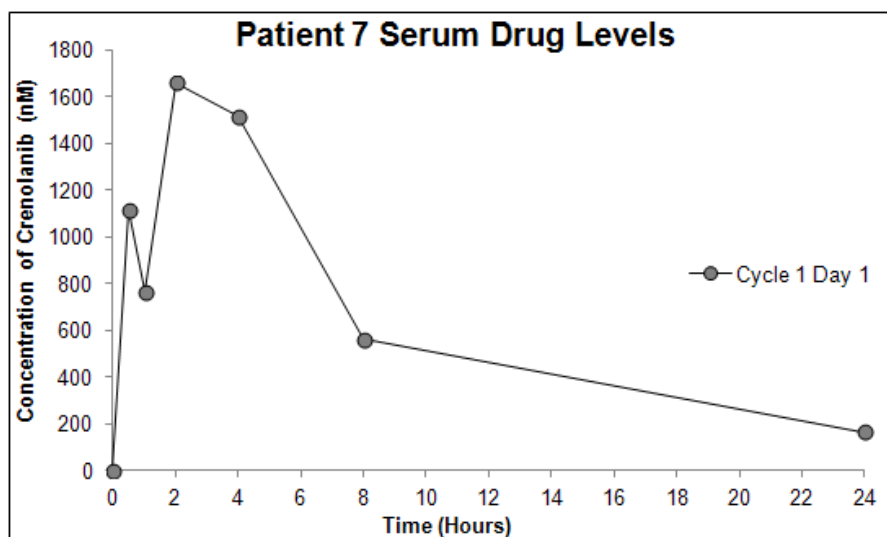
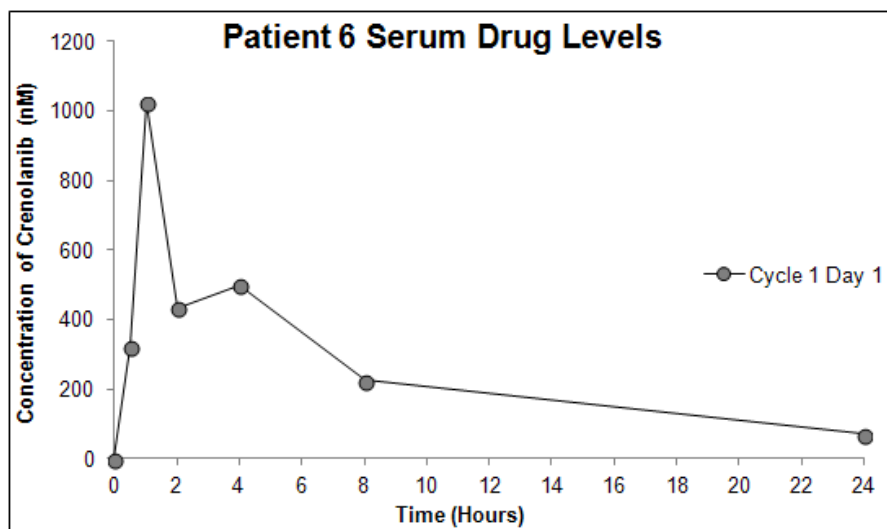
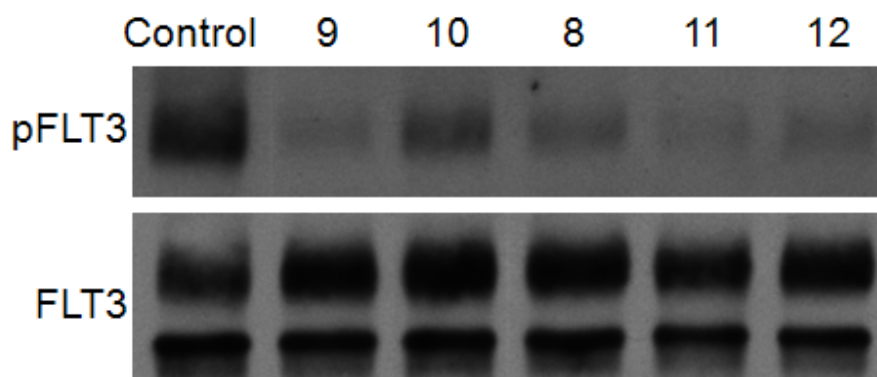


Figure 7B. Crenolanib inhibits FLT3 in vivo. The same time course samples from these patients, shown in 7A, were analyzed by mass spectroscopy for serum drug concentrations of crenolanib.



% pFLT3 inhibition	95	66	85	97	90
Serum crenolanib concentration (nM)	753	66	331	664	504

Figure 7C. Crenolanib inhibits FLT3 in vivo. Blasts from patient 4 (relapsed D835V) were utilized for a PIA assay with steady state plasma samples from five different patients enrolled on the crenolanib trial. Blasts were incubated in plasma for one hour, lysed, immunoprecipitated for FLT3, and analyzed by immunoblot for phospho- and total FLT3. Densitometry was performed on this blot and results are described under %FLT3 inhibition. Corresponding serum concentrations for the chosen sample are listed below.

Chapter 4

Bone marrow stroma-mediated resistance to FLT3 inhibitors

The FLT3 receptor is expressed in a limited number of tissues, mainly hematopoietic progenitor cells. However, its corresponding ligand, FL is expressed ubiquitously. Binding of FL to FLT3 induces dimerization of the receptor, autophosphorylation, and subsequent phosphorylation of downstream signaling molecules. Structural studies of ITDs in the juxtamembrane domain have shown that these mutations induce constitutive activation and ligand independent phosphorylation of the receptor.³² More recently, it has been shown that in FL knockout systems, the FLT3/ITD receptor shows only modest phosphorylation, but full phosphorylation when FL was re-introduced.³³ This demonstrates that FL still plays an important role in FLT3/ITD signaling despite the constitutive activation of the receptor. FL may play a role in a clinical setting where chemotherapy induces a dramatic rise in plasma FL levels.³⁴ The presence of FL has also been shown to reduce the efficacy of some FLT3 TKIs.³⁴ The tumor microenvironment has been shown to play a major role in many cancer types including AML^{35,36} and FL is expressed on the cell surface of bone marrow stromal cells which could reduce the efficacy of TKIs against AML blasts residing in the bone marrow. These observations demonstrate that effect of FL should be taken into account when developing new therapies. We hypothesized that monoclonal antibodies targeting FL would lessen the impact of stromal-mediated resistance to TKIs associated with stromal cell surface FL.

In Figure 8, blasts from two patients were treated with a FLT3 inhibitor in suspension culture and co-cultured with primary human bone marrow stroma. This experiment is designed to predict the effect of a FLT3 TKI on peripheral blood blasts, demonstrated by suspension culture, and the effect of a FLT3 TKI on blasts residing in

the bone marrow niche, demonstrated by the bone marrow stroma co-culture system. Quantification of this effect is displayed in Table 2. The stromal co-culture system causes a 4-6 fold shift in the IC₅₀ of crenolanib. This experiment reveals that the dose selected to treat a patient with a FLT3 TKI must be significantly higher to affect the blasts residing in the protective bone marrow niche than to simply eliminate circulating blasts.

To address this issue, we proposed targeting the bone marrow stromal cells to overcome their protective effect. We used novel anti-FL monoclonal antibodies produced by Symphogen (Lyngby, Denmark) with the aim of neutralizing receptor ligand binding and optimizing efficacy of FLT3 TKIs. Anti-FL mAbs 9449 and 9373 have no effect on WT FLT3 or FLT3/ITD phosphorylation and do not induce cytotoxicity in cell lines in suspension (Figure 9A and 9B). This shows that targeting FL alone has no effect on AML cells themselves. To test the effect of these mAbs in the context of the bone marrow microenvironment, primary blasts were incubated in parallel in suspension culture and stromal cell co-culture, with crenolanib, with or without the addition of mAb 9449 and analyzed by immunoblot (Figure 10). The addition of mAb 9449 is able to reduce the stromal shift of crenolanib IC₅₀ from 1.96 to 1.16 fold (Table 3). This highlights the potential for targeting FL to overcome stromal-mediated resistance to treatment with TKIs in AML.

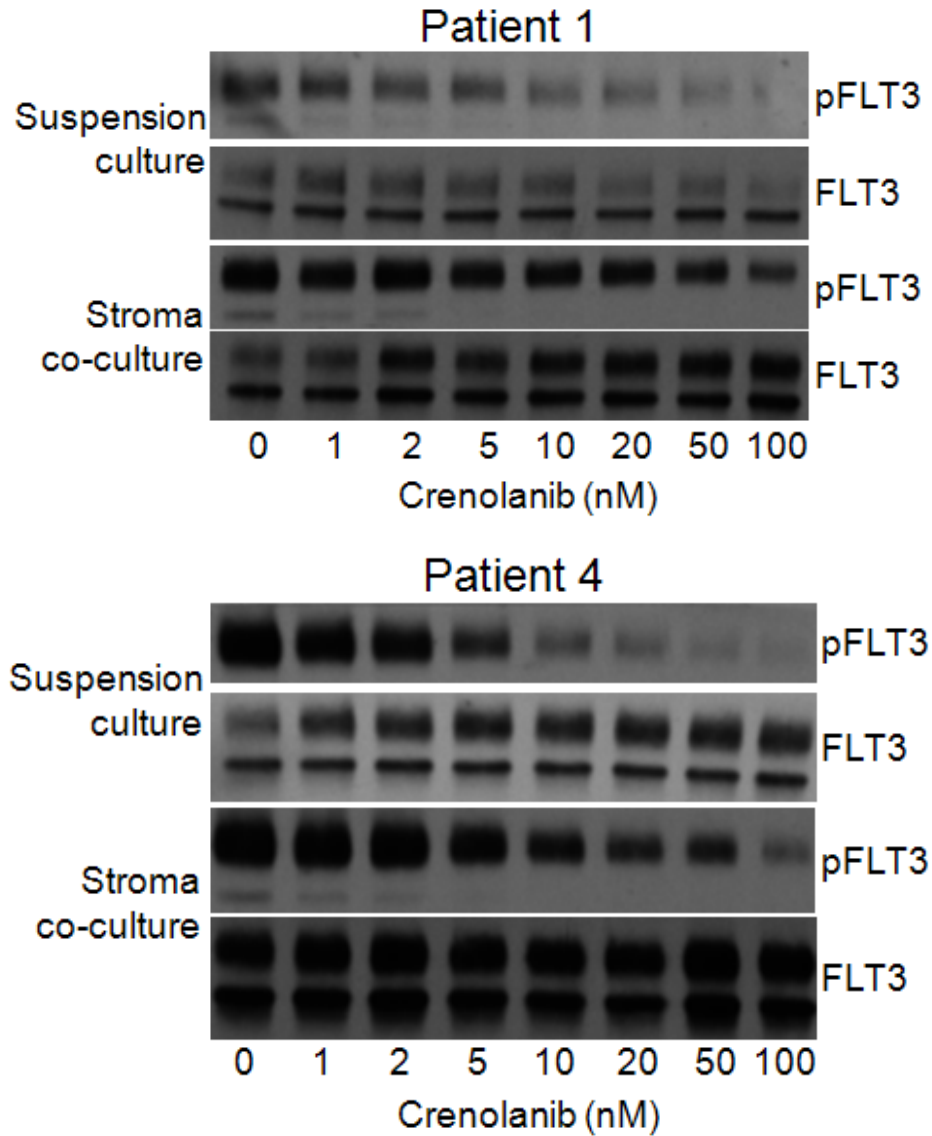


Figure 8. Stromal co-culture causes increased FLT3 phosphorylation. Blasts from patient 1 and patient 4 were treated with a dose response of crenolanib in two parallel conditions: in suspension culture or co-cultured with primary bone marrow stroma. Blasts were incubated with crenolanib in medium for one hour, lysed, immunoprecipitated for FLT3, and analyzed by immunoblot for phospho- and total FLT3.

Patient	FLT3 Mutation	Crenolanib pFLT3IC ₅₀	Fold change in IC ₅₀
Patient 1 (suspension)	ITD	2.39 nM	5.85
Patient 1 (stroma)	ITD	14 nM	
Patient 4 (suspension)	D835V	1.98 nM	3.99
Patient 4 (stroma)	D835V	7.91 nM	

Table 2. Stromal co-culture causes a shift in IC₅₀ of FLT3 TKIs. This table is a quantification of the immunoblots shown in Figure 8. Densitometry analysis was performed using Quantity One software (version 4.5.0). IC₅₀s were calculated by regression analysis after linear conversion (Calculusyn software).

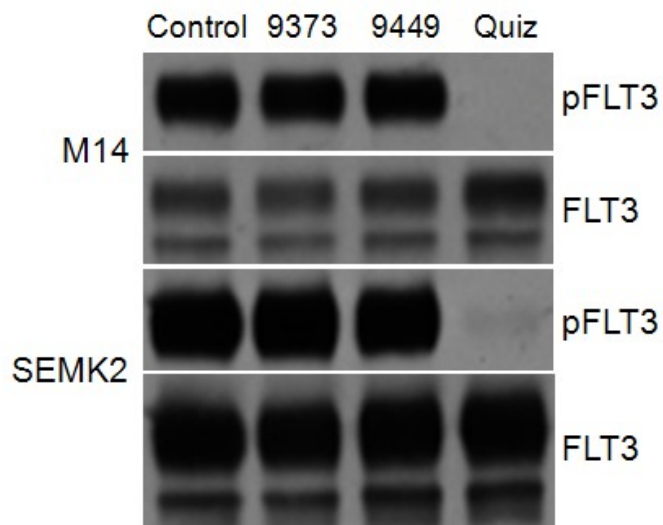


Figure 9A. Anti FL mAb effect on WT FLT3 and FLT3/ITD cell lines. Molm14 and SEMK2 cells were incubated with 100 μ g/mL of anti-FL mAb 9373, 9449, for two hours or 100nM quizartinib for one hour in medium, lysed, immunoprecipitated for FLT3, and analyzed by immunoblot for phospho- and total FLT3.

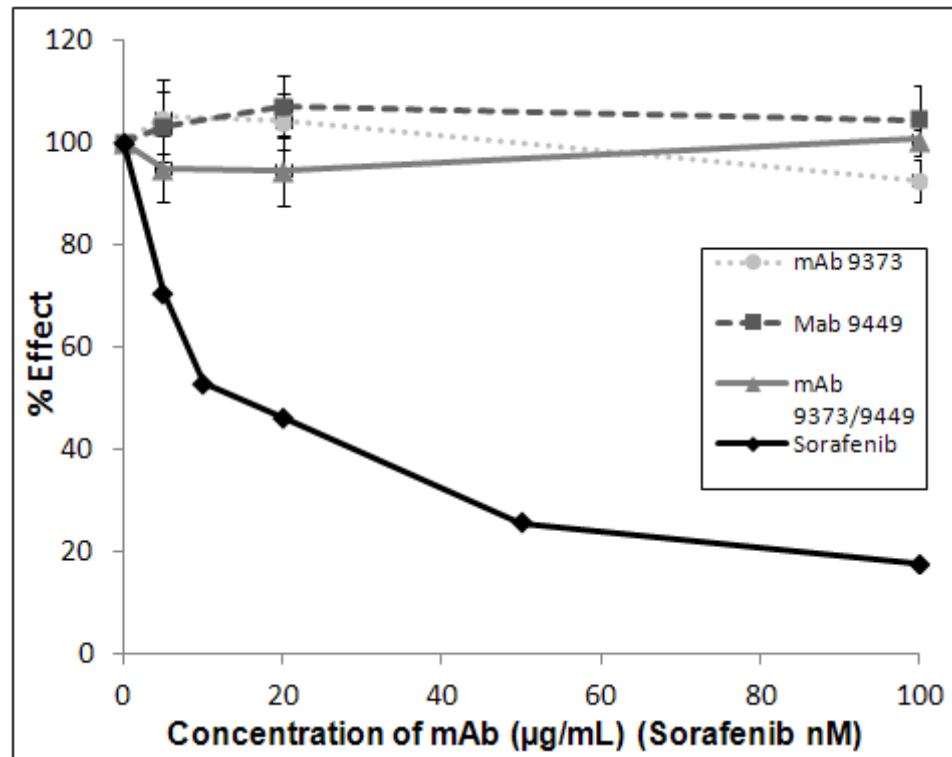


Figure 9B. Anti FL mAb effect on WT FLT3 and FLT3/ITD cell lines. Molm14 cells were treated with 100μg/mL of anti-FL mAb 9373, anti-FL mAb 9449, anti-FL mAb 9373 and 9449, or sorafenib in 96 well plates for 48 hours and cytotoxicity was analyzed by MTT.

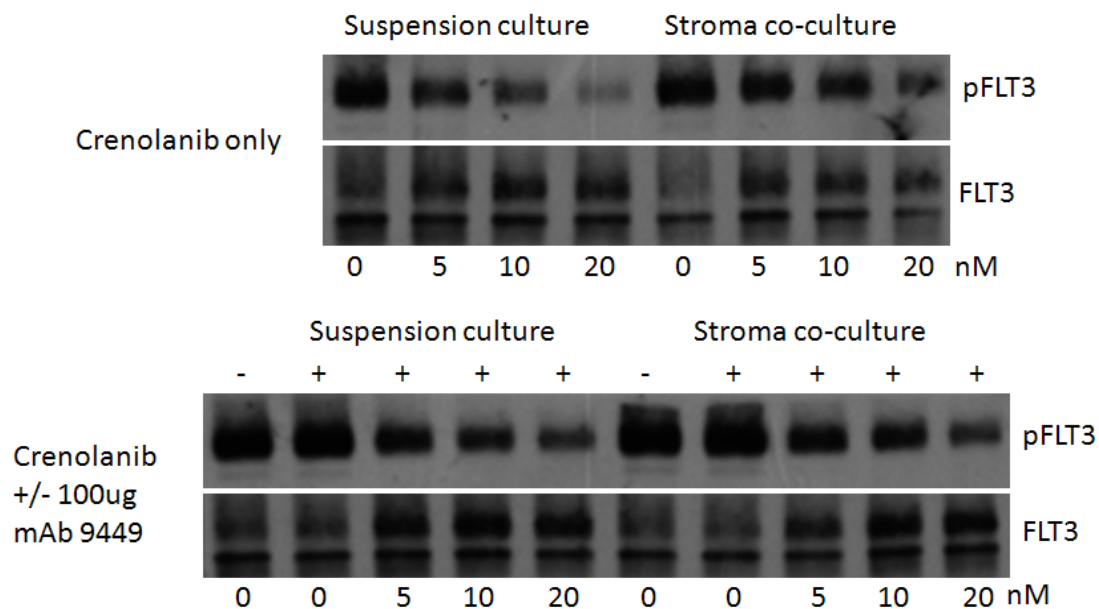


Figure 10. Stromal co-culture induced shift in TKI IC₅₀ is reduced by anti-FL mAbs. Primary patient blasts (patient 4) were treated with crenolanib for one hour in four parallel conditions. In the upper blot, blasts were incubated in suspension with crenolanib and co-cultured with stroma with crenolanib. In the lower blot, blasts were incubated in suspension with crenolanib and mAb9449 and co-cultured with stroma with crenolanib and mAb9449. Following one hour of drug treatment, cells were lysed, immunoprecipitated for FLT3 and analyzed by immunoblotting for phospho- and total FLT3.

Drug	Suspension culture IC₅₀	Stroma co-culture IC₅₀	Fold shift
Crenolanib only	5.58 nM	10.98 nM	1.96
Crenolanib + mAb 9449	5.25 nM	6.09 nM	1.16

Table 3. Stromal co-culture induced shift in TKI IC₅₀ is reduced by anti-FL mAbs.

Primary patient blasts were treated with crenolanib or crenolanib and mAb9449 in suspension or co-cultured with stroma for one hour, then cells were lysed, immunoprecipitated for FLT3 and analyzed by immunoblotting for phospho- and total FLT3 as shown in Figure 10. Densitometry analysis was performed using Quantity One software (version 4.5.0). IC₅₀s were calculated by regression analysis after linear conversion (Calculusyn software).

Chapter 5

Myelosuppression and off-target effects of tyrosine kinase inhibitors

Inhibition of c-Kit disrupts hematopoietic progenitor activity

Dasatinib is a multi-targeted kinase inhibitor with activity against Bcr-Abl, SRC, and c-Kit, among others. Using TF-1 cells stimulated with stem cell factor (SCF), we estimated that the concentration of dasatinib necessary to inhibit 50% of baseline c-KIT activity (IC_{50}) in an immunoblot assay is 1.5 nM in culture medium and 30 nM in 100% human plasma (Figure 11A). It has a relatively short half-life of 3-5 hours, and patients achieve maximum plasma concentrations of around 84 nM.³⁷ In colony formation assays with normal human bone marrow, dasatinib had a mild effect on the formation of myeloid and considerably inhibited the formation of erythroid colonies (Figure 11B). Dasatinib has been reported to cause myelosuppression in leukemia patients,²⁸ as well as hair depigmentation.³⁸ Pazopanib is reported to be a potent inhibitor of VEGFR, PDGFR, and c-Kit.³⁹ We found it to have an IC_{50} against c-Kit in culture medium of 3.7 nM. While pazopanib has a seemingly high IC_{50} in human plasma of 36 μ M due to high plasma protein binding (Figure 11A), many patients are able to achieve trough drug levels of 50 μ M.³⁹ In colony formation assays with normal human bone marrow, pazopanib caused substantial inhibition of erythroid colony formation and only moderate inhibition of myeloid colonies (Figure 11B). Hair depigmentation is listed as a common adverse event in the FDA label for pazopanib, indicative of in vivo c-Kit inhibition. While both dasatinib and pazopanib are potent in vitro inhibitors of c-Kit and both inhibit erythroid colony formation in vitro (and cause hair depigmentation), of the two drugs, only dasatinib has been reported to cause myelosuppression in vivo.^{28,39} indicating that, at least in the case of pazopanib, the progenitor cell assay does not fully replicate conditions in vivo. Pazopanib is used as a treatment for patients with solid tumors and is not used to treat hematological malignancies. One explanation for this result is that c-Kit inhibition may not induce clinically significant myelosuppression in the setting of normal bone marrow function.

FLT3 inhibition has no effect on hematopoietic progenitor activity

FLT3 and c-KIT are structurally related RTKs that both play important roles in early hematopoiesis.⁴⁰ In contrast to c-Kit, knockout of FLT3 is not embryonic lethal in murine transgenic models. Given their somewhat redundant signaling properties, there has been concern that simultaneous inhibition of FLT3 and c-KIT could result in more profound myelosuppression compared to inhibition of c-Kit alone. Sorafenib is a potent FLT3 TKI (IC₅₀ in culture medium 3-5 nM) that has demonstrated efficacy in the treatment of relapsed/refractory FLT3/ITD AML patients.⁶ There is no reported inhibition of c-Kit by sorafenib, nor have there been any reports of myelosuppression or hair depigmentation in clinical studies of this agent. These observations are consistent with the results of our immunoblot assays of c-Kit inhibition (Figure 11A) and by colony formation assays in which sorafenib showed no significant inhibition progenitor cell activity (Figure 11B). Quizartinib is a novel, potent FLT3 inhibitor (IC₅₀ in culture medium 2 nM; in plasma 18 nM) with a long half-life (1.5 days), and AML patients readily achieve micromolar plasma concentrations of quizartinib.⁸ Quizartinib is a modestly potent c-Kit inhibitor with an IC₅₀ in culture medium of 28 nM and myelosuppression was observed in clinical trials of quizartinib.³⁰ In colony formation assays, quizartinib inhibited both myeloid and erythroid colony formation, consistent with what we observed with dasatinib and pazopanib (Figure 11B). Quizartinib-induced myelosuppression, therefore, is probably mediated through inhibition of c-Kit.

It is still formally possible that while FLT3 inhibition by itself has no effect on hematopoiesis, it could contribute to c-Kit-induced marrow suppression. Exogenous FLT3 ligand (FL) is known to shift the dose response to FLT3 inhibitors upward. {Sato, 2011 #1013} If FLT3 inhibition were contributing to the suppression of colony formation induced by quizartinib, then the addition of FL to the methylcellulose medium would be predicted to blunt the inhibitory effect of quizartinib. In progenitor cell assays, we saw no significant difference in colony

formation with 200 nM quizartinib with or without exogenous FL (10 ng/mL) (data not shown), suggesting that FLT3 inhibition does not contribute to marrow suppression from quizartinib. In summary, our findings show that the more potent c-Kit inhibitors induce greater inhibition of erythroid colony formation and FLT3 inhibition alone appears to have minimal if any contribution to erythroid colony suppression without concomitant c-Kit inhibition.

Defining a therapeutic index

Table 4 lists several TKIs that we characterized according to their activity against both FLT3 and c-Kit receptors in culture medium and 100% human plasma. Each TKI was ranked according to our estimate of its relative potency against c-Kit in vivo, based on pharmacokinetic data, in vitro potency, the frequency of the clinical biomarker of hair depigmentation, and the frequency of myelosuppression observed in patients. Table 4 demonstrates a pattern of characteristics of TKIs that could be used to predict in vivo c-Kit inhibition. However, some TKIs do not strictly follow this pattern, such as pazopanib, which causes in vitro c-Kit inhibition and in vivo hair depigmentation but not myelosuppression in patients. This could be attributed to these TKIs having a variable range of activity against many kinases so it may be difficult to credit any one kinase alone with a given side effect.⁷ Clinicians treating patients with hematologic malignancies with c-Kit inhibitors must be aware of this therapeutic index in order to minimize the patient's risk of myelosuppression.

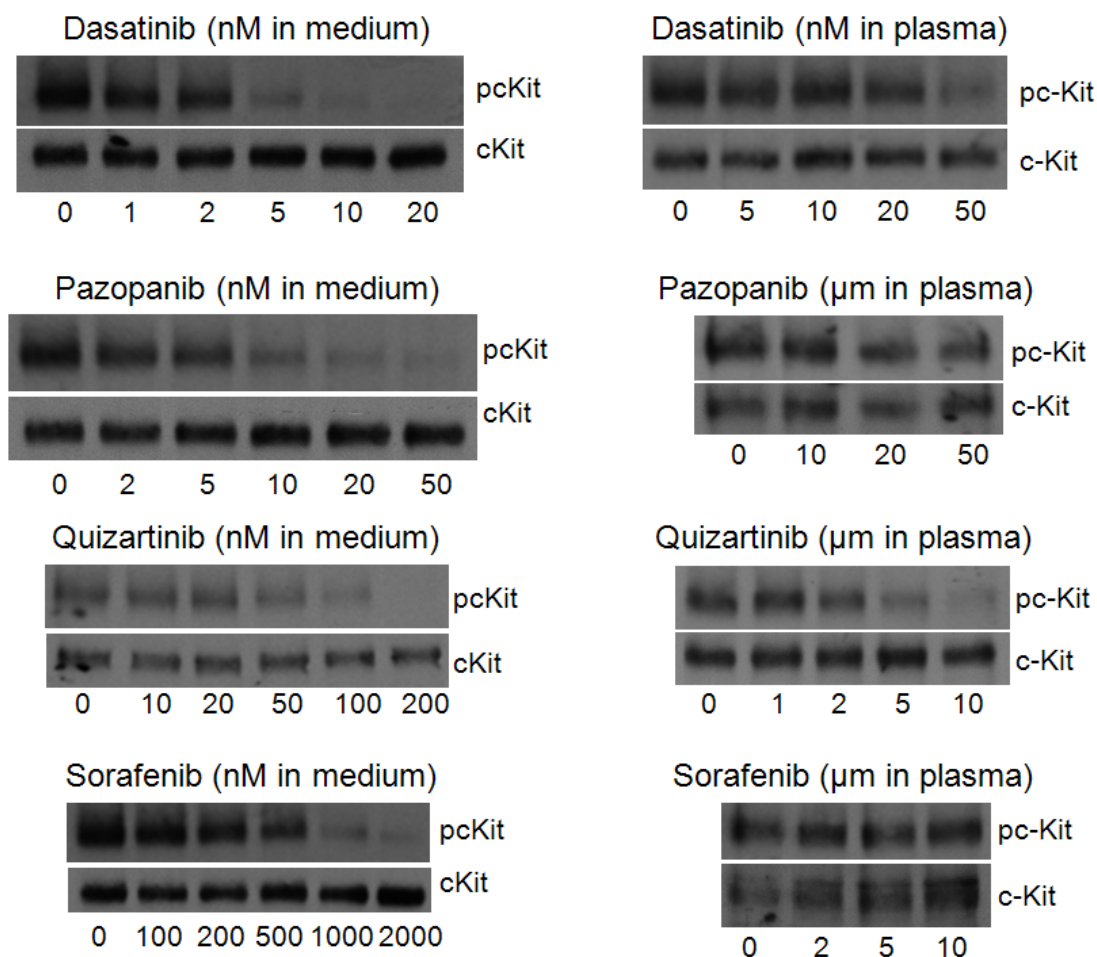


Figure 11A. c-Kit inhibition correlates with myelosuppression in vitro. TF-1 cells were treated with drug for 1 hour (20 ng SCF was added in the last five minutes of incubation) in cell culture medium or plasma, lysed, and immunoblotted for phospho- and total c-Kit.

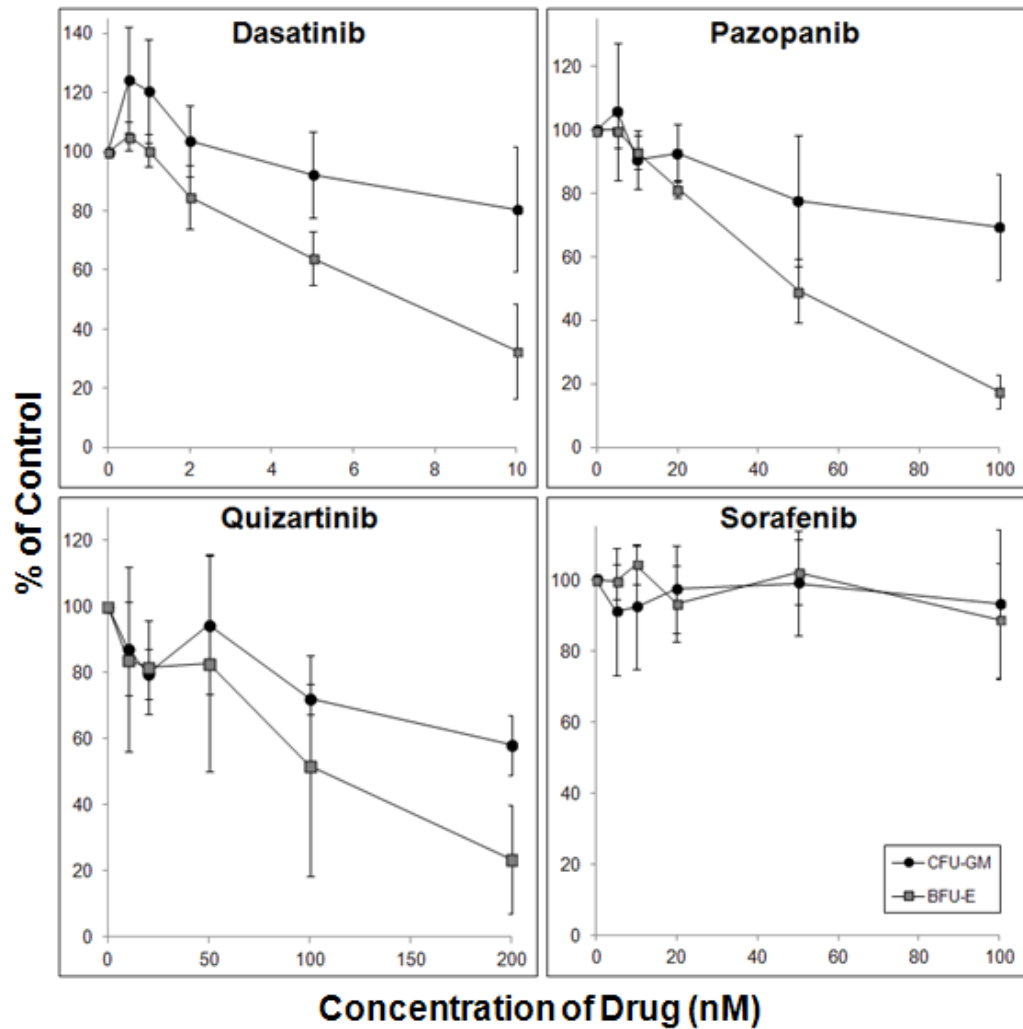


Figure 11B. c-Kit inhibition correlates with myelosuppression in vitro. Normal human bone marrow (n=3) was collected and mononuclear cells were isolated. Mononuclear cells were plated in 35 mm dishes at a concentration 100,000 cells per mL in MethoCult containing various concentrations of a TKI in quadruplicate. Plates were analyzed 10-14 days later by morphology for total number of CFU-GM and BFU-E colonies.

TKI	pFLT3 IC50 medium	pcKIT IC50 medium	pFLT3 IC50 plasma	pcKIT IC50 plasma	Hair Depig	Myelo- suppression	Half Life	Peak or trough levels in plasma	Ref
Sunitinib	9.6 nM	0.78 nM	136 nM	26 nM	Yes	Yes	41-86 hours	P: 135 nM T: 82 nM	41
Pazopanib	>2 μ M	3.7 nM	N/A	36 μ M	Yes	No	31 hours	P: 95 μ M T: 50 μ M	39
Quizartinib	2 nM	28 nM	18 nM	2.7 μ M	Yes	Yes	>1.5 days	P: N/A T: N/A	8
Dasatinib	>200 nM	1.5 nM	N/A	30 nM	Occasional	Yes	3-5 hours	P: 84 nM T: N/A	37
Imatinib	>2 μ M	122 nM	N/A	5.4 μ M	Occasional	Yes	19 hours	P: 4.7 μ M T: 1.9 μ M	42
Crenolanib	2.6 nM	62 nM	48 nM	2.0 μ M	No	No	12 hours	P: 2.8 μ M T: N/A	19
Sorafenib	5.2 nM	285 nM	484 nM	>10 μ M	No	No	25-48 hours	P: 12.9 μ M T: 8 μ M	43, 44

Table 4. Ranking of a panel of TKIs based on predicted potency against c-Kit in

vivo. For FLT3 data, MOLM14 cells were incubated with drug for 1 hour, lysed, immunoprecipitated for FLT3, and immunoblotted for phospho- and total FLT3. For c-Kit data, TF-1 cells were incubated with drug for 1hr with 20 ng SCF (PeproTech, Rocky Hill NJ) added to each sample in the last 5 minutes of drug incubation. The treated TF-1 cells were lysed, immunoprecipitated for c-Kit, and immunoblotted for phospho- and total c-Kit. Densitometry analysis was performed using Quantity One software (version 4.5.0). IC50s were calculated by regression analysis after linear conversion (Calculusyn software).

Chapter 6

Significance and future directions

FLT3 inhibitors have been under investigation for over a decade. First generation inhibitors, such as lestaurtinib, midostaurin, and sunitinib, were re-purposed multi-targeted compounds with minimal clinical activity as single agents because they lacked potency and/or tolerability. Second generation inhibitors such as quizartinib and PLX3397 are more selective, and therefore presumably better tolerated at concentrations necessary to fully inhibit FLT3 in vivo. This has led to more impressive clinical results, particularly with quizartinib,^{8,45} but has also not unexpectedly led to the emergence of resistance-conferring point mutations, most commonly at residue D835. The emergence of these mutations in the context of FLT3 inhibition represents evidence that FLT3 mutations- either the ITD mutation at diagnosis or the ITD/D835 combination- represent driver mutations for this disease. Furthermore, while isolated FLT3/D835 mutations (i.e., those occurring in the absence of a FLT3/ITD mutation) overall have an uncertain prognostic impact, in some cases they probably also function as driver mutations (e.g., see Figure 3), and therefore warrant targeting.⁴⁶

Crenolanib has been studied in patients with solid tumors,¹⁹ and is currently being studied in patients with PDGFR-driven gastrointestinal stromal tumors (NCT01243346) and in pediatric patients with gliomas (NCT01393912), as well as in AML patients with FLT3 activating mutations (NCT01522469; NCT01657682). Preliminary clinical and pharmacokinetic data from these studies indicate that the drug is cytochrome P450-metabolized, has an approximate terminal half-life ($t_{1/2}$) of 8 hours, and is well-tolerated at 100 mg three times daily (Arog Pharmaceuticals, *personal communication*). This $t_{1/2}$ is considerably shorter than that of sorafenib (~24 hours) or quizartinib (1.5 days),^{8,47} necessitating thrice daily dosing.

Crenolanib has no activity against the F691 point mutations, which also emerged during quizartinib, albeit at a lower frequency than the D835 mutations, and we did not test the drug exhaustively against all of the other relatively uncommon FLT3 point mutations that have been previously reported.^{18,46,48-50} It is likely that as this field progresses, a number of compounds will emerge that have overlapping activity against FLT3 variants,^{51,52} similar to the case with inhibitors of BCR-ABL. However, FLT3/ITD and FLT3/D835 mutations, either arising spontaneously or in response to treatment with a FLT3TKI, constitute the vast bulk of clinically important FLT3 activating mutations, and we have demonstrated here that crenolanib not only has in vitro activity against them, oral dosing of the drug can achieve inhibitory concentrations against these mutations in AML patients.

Myelosuppression can be a challenging clinical parameter to evaluate in any trial involving AML patients. Most AML patients enrolled on early phase studies are already myelosuppressed from their disease burden. Nonetheless, suppression of bone marrow function to some degree is almost always a feature of TKIs that have activity against c-KIT, and the relatively limited activity of crenolanib against c-KIT may offer a unique advantage of this drug over others in this class.

In summary, our results indicate that crenolanib represents the next generation of FLT3 TKIs, one with a broader range of activity than the other agents in development. Phase 2 trials of this drug in FLT3-mutated AML patients are ongoing (NCT01657682 and NCT01522469), and phase 3 trials are currently in the planning stage.

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Educational History:

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Baltimore, MD
Ph.D. Cellular and Molecular Medicine

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B.S. Biology: Biomedical Concentration

Professional Experience

- Adjunct Faculty, Towson University, Department of Biological Sciences (2013)
- Research Rotation, Lab of Dr. Robert Getzenberg, Johns Hopkins School of Medicine (2010)
- Research Rotation, Lab of Dr. Elizabeth Jaffee, Johns Hopkins School of Medicine (2010)
- Course Assistant, Frontiers in Reproduction, Woods Hole Marine Biological Lab (2010)
- Undergraduate Research, Lab of Dr. Jeffery Bowen and Dr. Merideth Krevosky, Bridgewater State University (2008-2010)
- Summer Research Intern, Lab of Dr. Meredith Gregory-Ksander, Schepens Eye Research Institute (2009)
- Laboratory Teaching Assistant, CityLab, Bridgewater State University (2009)

Academic Honors

- Towson University/Johns Hopkins Teaching Fellow, Johns Hopkins University School of Medicine and Towson University (2013)
- 29 Who Shine on Massachusetts Public College and University Campuses, Bridgewater State College (2010)
- Dean's List, Bridgewater State University (2006-2010)
- Beta Beta Beta National Biological Honor Society (Member 2008-2009, President BSU Chapter 2009-2010)
- Honors Program, Bridgewater State University (2006-2008)

Publications

Galanis A, Ma H, Rajkhowa T, Ramachandran A, Small D, Cortes J, Levis M. Crenolanib is a potent inhibitor of FLT3 with activity against resistance-conferring point mutants. *Blood*. 2014; 123(1): 94-100.

Posters and Abstracts

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Activities of Commonly Used Tyrosine Kinase Inhibitors Against Wild-Type C-KIT and Potential Impact On Normal Hematopoiesis. **Allison Galanis**, Mark J. Levis.
- American Society of Hematology Annual Meeting (December 2012)
Crenolanib Is A Highly Potent, Selective, FLT3 TKI with Activity Against D835 Mutations. **Allison Galanis**, Trivikram Rajkhowa, Chaitanya Muralidhara, Abhijit Ramachandran and Mark J. Levis.
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Crenolanib: A next generation FLT3 inhibitor. **Allison Galanis**, Trivikram Rajkhowa, Chaitanya Muralidhara, Abhijit Ramachandran, Mark Levis.
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Role of Survivin and small heat shock proteins in the chemoresistance of retinoblastoma. Margaret Koch, Jeffery A. Bowen, **Allison Galanis**, Meredith Gregory, Bruce R. Ksander, Merideth K. Krevosky.
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The Novel Inhibitor PLX3397 Effectively Inhibits FLT3-Mutant AML. Elizabeth Burton, Bernice Wong, Jiazhong Zhang, Brian West, Gideon Bollag, Gaston Habets, **Allison Galanis**, Hoa Nguyen, Omotayo Arowojolu, Trivikram Rajkhowa and Mark J. Levis.
- Association for Research in Vision and Ophthalmology Annual Meeting (May 2010)
Chemotherapy-Dependent Upregulation of Small Heat Shock Proteins in Retinoblastoma: Do They Mediate Chemoresistance? M Kamradt Krevosky, **AE Galanis**, JA Bowen, E Whiston, N Sugi, M Gregory, BR Ksander.
- National Conference on Undergraduate Research (April 2010)
Inhibition of Chemotherapy-Induced Apoptosis by Heat Shock Proteins in Retinoblastoma. **Allison Galanis**, Jeffery Bowen and Merideth Krevosky.
- Eastern New England Biological Conference (April 2010)
Regulation of Heat Shock Proteins in Etoposide Treated Retinoblastoma Cells. **Allison Galanis**, Jeffery Bowen and Merideth Krevosky.
- Association for Research in Vision and Ophthalmology Annual Meeting (May 2009)
Does α B-crystallin mediate chemoresistance in retinoblastoma? M Kamradt Krevosky, **AE Galanis**, JA Bowen, E Whiston, N Sugi, M Gregory, BR Ksander.